Multiple Oxygenase Reactions in the Biosynthesis of Taxoids

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Received April 20, 1998

Taxoids are polycyclic diterpenes produced by various yew species (for review see ref 1). Two compounds, the naturally occurring Taxol and the semisynthetic Taxotere, are potent antitumor agents acting via the stabilization of microtubuli.[2,3] The supply crisis for Taxol and naturally occurring taxoids has stirred the interest to produce these compounds biosynthetically. A full understanding of the taxoid biosynthetic pathway is necessary to provide the basis for an optimized biotechnological production of taxoids. The isoprenoid precursors of taxoids, dimethylallyl pyrophosphate (DMAPP, **3**) and isopentenyl pyrophosphate (IPP, **2**), are formed via 1-deoxy-D-xylulose 5-phosphate (**1**, Figure 1).[4] It is generally accepted that DMAPP and three molecules of IPP are condensed to yield geranylgeranyl pyrophosphate (**4**). The generation of the polycyclic taxane precursor, taxa-4(5),11(12)-diene (**5**), from **4** is catalyzed by a single enzyme.[5] Cyclization is followed by introduction of a hydroxyl group in position 5-α yielding **6**. This reaction is catalyzed by a mixed function cytochrome P_{450} dependent hydroxylase with use of molecular oxygen as substrate.[6] The acylation of the 5-hydroxy group yielding **7** has been described as a subsequent step in the taxoid pathway.[7] The introduction of additional hydroxyl functions at various positions of the taxane ring system and their subsequent esterification yields a variety of natural products. The mechanisms and the enzymes catalyzing these reactions are still unknown.

The biosynthesis of taxoids can be studied conveniently in a cell culture of *Taxus chinensis* which produces taxoids in 4% (dry weight) yield.[8] The most abundant taxoids produced by the cell line are 2α,5α,10β,14β-tetraacetoxy-4(20),11-taxadiene (taxuyunnanine C, **8**) and 2α,5α,10β-triacetoxy-14β-(2′-methyl-3′-hydroxy)butyryloxy-4(20),11-taxadiene (yunnanxane, **9**).[8] To determine the biogenetic origin of the various oxygen functions in **8** and **9**, we cultured a suspension of *T. chinensis* cells in an atmosphere enriched with $^{18}O_2$. The carbon source in the culture medium was a mixture of [1-^{13}C]glucose and unlabeled glucose (molar ratio, 1:4). As shown earlier, this feeding strategy yields metabolites with increased ^{13}C content in positions 2, 6, 10, 14, 16, 18, 19, and 20 of the taxoid ring system.[4] The ^{13}C labeling served to increase the sensitivity of ^{13}C NMR analysis of the biosynthetic taxoids.

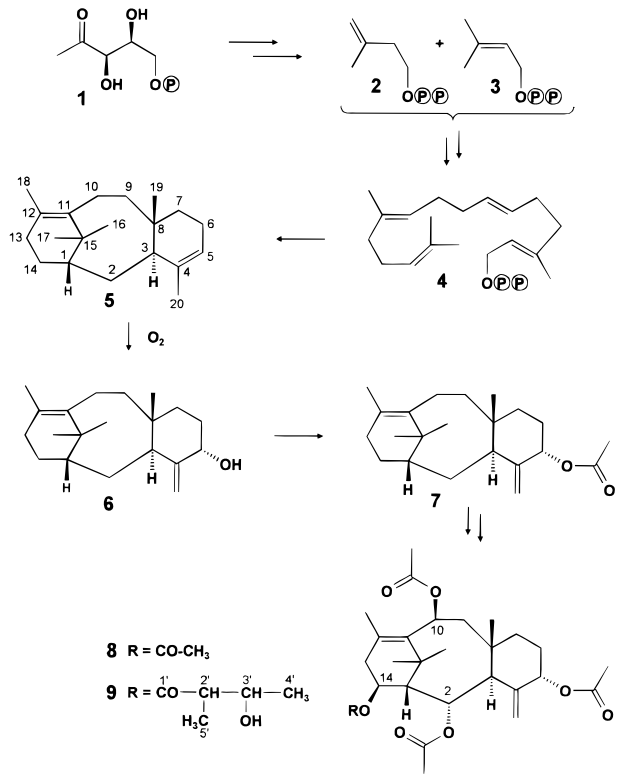

Figure 1. The taxoid biosynthetic pathway.

The heterotrophic metabolism of cultured plant cells generates large amounts of CO_2. To remove CO_2 from the ^{18}O-enriched atmosphere, the cells were grown in a 2000 mL shaking flask containing 400 mL of the cell suspension and 100 mL of 1.5 M sodium hydroxide in a separate compartment. Oxygen consumed by the cell culture was replaced by $^{18}O_2$ from an isobaric gasometer. The culture was incubated at 24 °C with shaking (100 rpm). After a culture period of 10 days, the cell culture had consumed 3.5 L of $^{18}O_2$ (98% ^{18}O enrichment). The cells (5.2 g, dry weight) were harvested, and taxuyunnanine C (20 mg) and yunnanxane (1.4 mg) were isolated as described earlier.[4,8]

^{13}C NMR signals of taxayunnanine C (**8**) and yunnanxane (**9**) are shown in Figure 2. ^{13}C NMR and ^{1}H NMR signal assignments for the compounds under study have been reported earlier.[4,8] The ^{13}C signals of the carbon atoms shown in Figure 2 have upfield shifted satellites (marked by arrows) accounting for 7−11% of the signal. Similarly, the carboxy carbons of the acetoxy residues bound to these carbon atoms show upfield shifted satellites in the ^{13}C NMR spectrum of taxuyunnanine C (**8**) (Figure 2A, Table 1). For sensitivity reasons no upfield shifted satellites could be detected in the carboxy carbon atoms of yunnanxane (**9**). The relatively low ^{18}O abundance in the metabolites can be attributed to the following factors: (i) the gas phase was a mixture of air supplemented with $^{18}O_2$ and oxygen was depleted by the culture, and (ii) a substantial amount of taxoids was already present in the culture when $^{18}O_2$ supplementation was initiated.

The magnitudes of the putative ^{18}O isotope shifts (Table 1) were similar in structurally related compounds.[9] No upfield satellites were associated with any other taxane ring ^{13}C signals of **8** and **9**. In addition to the ^{18}O upfield shifted signal (marked

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S0002-7863(98)01353-5 CCC: $15.00
Published on Web 09/09/1998

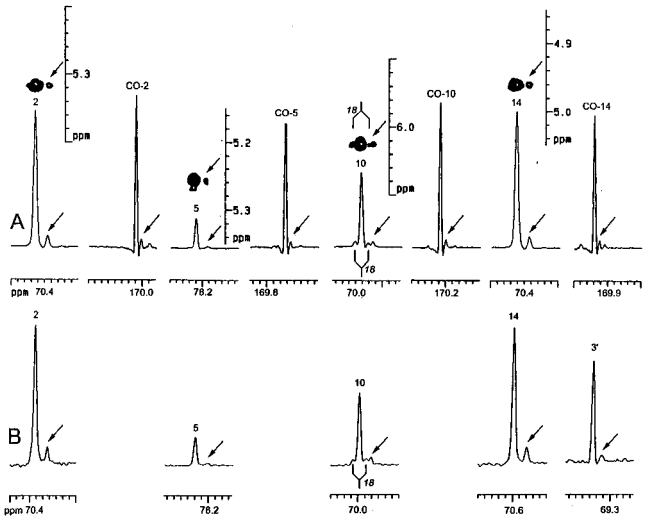

Figure 2. ^{13}C NMR signals of taxuyunnanine C (**8**, A) and yunnanxane (**9**, B) from *T. chinensis* after growth with [1-^{13}C]glucose under an atmosphere containing $^{18}O_2$. Satellite signals upfield shifted by ^{18}O-isotope effects are indicated by arrows. On top of ^{13}C signals of taxuyunnanine C, the corresponding strips from a two-dimensional $^{13}C^1H$-COSY experiment are shown. ^{13}C satellite signals of C-10 arising from long-range $^{13}C^{13}C$ coupling to C-18 are indicated.

Table 1. ^{13}C NMR Analysis at 125.7 MHz of Taxuyunnanine C (**8**) and Yunnanxane (**9**) from a Cell Culture of *T. chinensis* under an Atmosphere Containing $^{18}O_2$

	chemical shift[a] ppm		isotope shift[b] $\Delta^{13}C$ (^{18}O), ppb		isotopomer composition[c] [$^{18}O_1$], %	
position	**8**	**9**	**8**	**9**	**8**	**9**
2	70.44	70.39	36[d]	36	7[d]	11
5	78.16	78.12	33	35	8	8
10	69.98	69.97	35	35	nd[f]	nd[f]
14	70.44	70.62	36[d]	36	7[d]	11
3′		69.36		25		7
Ac2-CO	170.02	169.86	14	nd[e]	4	nd[e]
Ac5-CO	169.75	169.70	14	nd[e]	3	nd[e]
Ac10-CO	170.23	170.14	14	nd[e]	5	nd[e]
Ac14-CO	169.95	174.67	14	nd[e]	4	nd[e]

[a] Chemical shifts are referenced to the $CDCl_3$ signal at 77.0 ppm. [b] An upfield shift in the ^{13}C NMR chemical shift upon ^{18}O isotopic substitution is given as a positive value. [c] Referenced to the sum of the ^{13}C NMR signal integrals of each carbon atom. [d] Mean values due to signal overlapping. [e] nd, not determined due to weak signal intensities. [f] nd, not determined due to signal overlapping.

by arrow) the ^{13}C NMR signals of C-10 in **8** and **9** gave $^{13}C^{13}C$ coupled satellites which were conducive to ^{13}C coupling via three bonds to methyl carbon atom C-18 ($^3J_{CC}$ = 4.7 Hz). It should be noted that C-18 acquired ^{13}C label from [1-^{13}C]glucose and long-range ^{13}C coupling to C-10 was well resolved due to the relatively large $^3J_{CC}$ coupling via the sp^2-hybridized carbon atoms C-11 and C-12.

The ^{13}C NMR signals of C-2, C-5, C-10, and C-14 of **8** were further analyzed by a two-dimensional $^{13}C^1H$-COSY experiment (Figure 2). Both the major signals as well as the upfield-shifted satellites gave identical correlation peaks to H-2, H-5, H-10, and

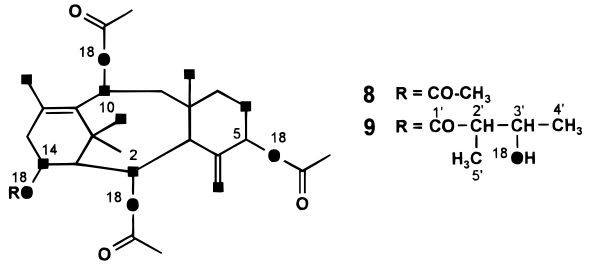

Figure 3. Labeling pattern of taxuyunnanine C (**8**) and yunnanxane (**9**) from *T. chinensis* after growth with [1-^{13}C]glucose under an atmosphere containing $^{18}O_2$. A solid square indicates ^{13}C enrichment from [1-^{13}C]-glucose.[4]

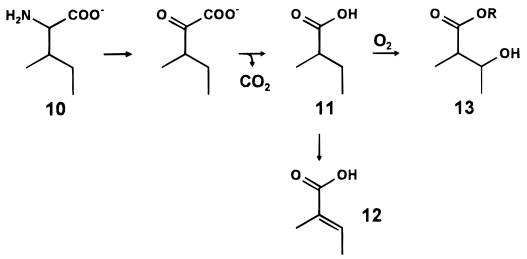

Figure 4. Hypothetical biosynthesis of the 3-hydroxy-2-methylbutyric acid moiety in yunnanxane.

H-14 signals, respectively, thus confirming that the satellite signals are due to ^{18}O labeled isotopomers of the taxoid. We conclude that all oxygen atoms attached to the taxoid ring systems of **8** and **9** are biosynthetically introduced from molecular oxygen. The introduction of oxygen into the taxane ring is thus likely to be catalyzed by cytochrome P_{450}-type monooxygenases. The carbonyl oxygens of the acetyl residues were apparently derived from glucose, proffered via the medium in large excess.

The acyl side chain at position 14 of **9** carries a 3′ hydroxy group. The ^{13}C NMR signal of the side chain carbon atom 3′ shown in Figure 2 is characterized by an upfield-shifted satellite (marked by an arrow) accounting for 7% of the integral signal intensity indicating the formation of the hydroxylated acyl side chain by an oxygenase reaction similar to the oxygenation of the taxane ring system. We propose that the 2-methyl-3-hydroxybutyryl ester group (**13**) of yunnanxane is derived from 2-methylbutyric acid (**11**), which is known to be formed via isoleucine (**10**) as an intermediate in the biosynthetic pathway of angelica acid (**12**) in plants (Figure 4).[10]

It has been shown earlier in vitro that the hydroxy function at C-5 of the taxoid ring is introduced from molecular oxygen by a cytochrome P_{450} dependent monooxygenase.[6] This is confirmed by our in vivo experiment. Moreover, we present evidence that the oxygen functions at C-2, C-10, and C-14 of the taxoid ring are introduced by molecular oxygen thus indicating the central role of monooxygenase-catalyzed reactions in the transformation of the taxane ring system.

Acknowledgment. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 369) and the Fonds der Chemischen Industrie.

JA981353P

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